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SYNTHESIS OF DOUBLY 15-NITROGEN LABELLED N-[4-HYDROXYBUTYL]-N-NITROSOBUTYLAMINE, A POTENT URINARY BLADDER CARCINOGEN IN ANIMALS

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SUMMARY

The labelled precursors for the amino nitrogen atom was ^{15}N-potassium phthalimide which was condensed with triphenylmethyloxychlorobutane. The phthalimide group was then cleaved with hydrazine and the primary amine obtained was condensed with butyraldehyde followed by sodium borohydride reduction to give the secondary amine N-[4-triphenylmethyloxybutyl]butyl-[^{15}N]amine. After deprotection of the triphenylmethyl group with trifluoroacetic acid, the amino alcohol obtained was nitrosated with ^{15}N-sodium nitrite in hydrochloric acid medium to give N-[4-hydroxybutyl]-N-[^{15}N]nitrosobutyl[^{15}N]amine. The two 15-nitrogen atoms were incorporated in order to obtain a tool useful for studying the metabolic activation of this compound. The evolution of labelled molecular nitrogen will be used to quantify the extent of nitrosamine hydroxylation at the carbon atom α to the nitroso moiety.

Key Words: N-[4-hydroxybutyl]-N-[^{15}N]nitrosobutyl[^{15}N]amine, nitrosamine, 15-nitrogen labelling, chemical carcinogen.

INTRODUCTION

Among known N-nitroso compounds, N-[4-hydroxybutyl]-N-nitrosobutylamine (1) and its precursor N-dibutyl-N-nitrosamine selectively induce urinary bladder tumors in laboratory animals [1]. The carcinogenic activity of these two compounds seems to depend on formation of their common metabolite N-[3-carboxypropyl]-N-nitrosobutylamine [2]. However, recent studies have indicated that the latter might not be responsible for urinary bladder tumor induction, suggesting that other metabolic pathways are involved in the activation of 1 to a carcinogen [3].

The biotransformation of these two products has been extensively investigated and the metabolites retaining the nitroso moiety have been characterized [2]. However, the metabolic

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activation of a chemical to a carcinogen involves the formation of labile highly reactive electrophilic intermediates interacting with cellular macromolecules. The most widely accepted metabolic pathway leading to the formation of such entities from nitrosamines is shown in Fig. 1. The first step involves an enzymatic hydroxylation at the carbon atom α to the nitroso group; all subsequent steps are non-enzymatic reactions and the final result is the formation of a carbocation and molecular nitrogen in stoichiometric quantity [4, 5].

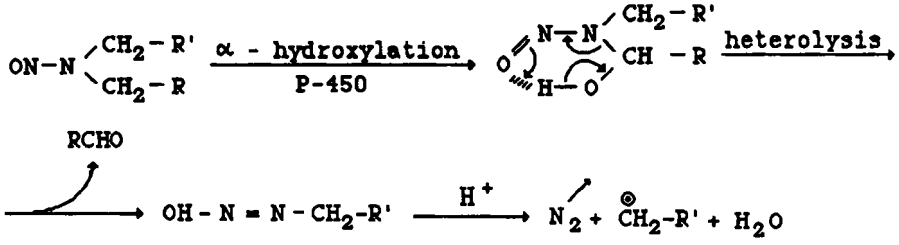

Fig. 1. Metabolic activation of nitrosamines.

Quantitative determination of molecular nitrogen evolved during the metabolism of nitrosamines has been used as an indicator of α-hydroxylation [6, 7]. Measurement of N_2 by gas chromatography requires extensive bubbling of the reaction mixture and head space gas with helium or oxygen to purge them of dissolved nitrogen [6]. The use of doubly ^{15}N labelled nitrosamines and the detection of $^{15}N_2$ by mass spectrometry make it simpler to measure the extent of α-hydroxylation and add specificity to the method [7].

The present paper reports the synthesis of N-[4-hydroxybutyl]-N-[^{15}N]nitrosobutyl-[^{15}N]amine (2). Using the compound labelled with the ^{15}N stable isotope helps gain a better understanding of the mechanism by which it exerts its carcinogenic effect.

EXPERIMENTAL

^{15}N-Potassium phthalimide, 99 atom % and ^{15}N-sodium nitrite, 99 atom % were purchased from MSD Isotopes, Montreal, Canada. All other chemicals were of analytical reagent grade. The identity of the compounds was established by ^1H NMR, ^{15}N NMR and MS. Proton and ^{15}N NMR spectra were recorded on a Varian VXR-300 NMR spectrometer operating at 299.9450 and 30.4060 MHz respectively. Mass spectra were determined on a VG TS 250 mass spectrometer in the electron impact (EI) mode (electron energy 30 eV), or chemical ionization (CI) mode using isobutane as reagent gas. Samples were introduced by the direct inlet system.

1-Chloro-4-triphenylmethyloxybutane (3)

A solution of 3.255 g (30 mmoles) of 4-chloro-1-butanol and 8.355 g (30 mmoles) of triphenylmethyl chloride in 50 ml of dry pyridine was stirred at room temperature under nitrogen for 3 days. Pyridine was removed in vacuo and the residue was extracted with diethyl ether, dried over anhydrous magnesium sulfate, filtered and evaporated to dryness. The crude product was chromatographed on a silica gel column with hexane-ethyl acetate 9:1 as eluting solvent, to give 8.41 g (80%) of amorphous white powder; TLC in 7:3 hexane-ethyl acetate (Rf 0.55); ^{1}H NMR ($CDCl_3$) δ 7.50-7.20 (m, 15H, aromatic protons); 3.52 (t,J = 6.2 Hz, 2H, $ClCH_2$); 3.10 (t, J = 6.3 Hz, 2H, OCH_2); 1.90-1.70 (m, 4H, $2CH_2$); mass spectrum, m/z (relative intensity) 352 (7.8), 350 (21), 273 (17), 259 (3), 243 (100), 183 (12), 165 (28), 105 (41), 91 (39).

N-[4-triphenylmethyloxybutyl][^{15}N]phthalimide (4)

A stirred mixture of compound 3 (3.505 g, 10 mmoles) and ^{15}N - potassium phthalimide (1.85 g, 10 mmoles) in 20 ml dimethylformamide (DMF) was heated at 140-150°C under an atmosphere of dry nitrogen for 2 h. After cooling, the solvent was evaporated in vacuo to dryness. Water (20 ml) was added to the residue and extracted with methylene chloride (2 x 50 ml). The organic layer was washed with 5% aqueous potassium hydroxide followed by water, dried and evaporated to give crystals which were recrystallized from isopropanol (4.15 g; 90%), m.p. 127-128°C; TLC in 1:1 hexane-ethyl acetate (Rf 0.5); ^{1}H NMR (*) ($CDCl_3$) δ 7.80-7.20 (m, 19H, aromatic protons); 3.70 (t, J = 7.1 Hz, 2H, NCH_2; 3.8 (t, J = 6.3 Hz, 2H, OCH_2); 1.90-1.60 (m, 4H, $2CH_2$); mass spectrum, m/z (relative intensity) 462 (0.5), 385 (4), 259 (21), 243 (100), 219 (46), 203 (32), 161 (48), 105 (16), 71 (7).

N-[4-triphenylmethyloxybutyl][^{15}N]amine (5)

A mixture of compound 4 (4.61 g, 10 mmoles), absolute ethanol (250 ml) and hydrazine hydrate (3.4 ml, 70 mmoles) was stirred at reflux for 15 h. The solid precipitate (phthalazine) was filtered off and then the mixture was evaporated to a semisolid residue. This was washed in a separatory funnel with methylene chloride (220 ml) and 0.3 N sodium hydroxide (250 ml). After shaking the solution, the organic phase was separated, washed with water (250 ml), dried and evaporated to give the amine 5 as a clear gum in 90% yield. ^{1}H NMR ($CDCl_3$) δ 7.45-7.20 (m, 15H, aromatic protons); 3.07 (t, J = 6.5 Hz, 2H, OCH_2); 2.68 (t, J = 7.0 Hz, 2H, NCH_2); 1.90 (broad s, 2H, NH_2); 1.70 - 1.48 (m, 4H, $2CH_2$); mass spectrum, m/z (relative intensity), 332 (0.5), 260 (11), 243 (100), 183 (26), 165 (57), 105 (27), 89 (87), 73 (20), 71 (29).

(*) Unless otherwise specified, ^{1}H NMR spectra are referred to unlabelled compounds.

N-[4-triphenylmethyloxybutyl]butyl[^{15}N]amine (6)

Butyraldehyde (3.74 ml, 41.5 mmoles) was added to a solution of 5 (2.75 g, 8.3 mmoles) in 150 ml hexane. The mixture was heated to reflux and the water produced was azeotropically distilled off. The solution was evaporated and the residue was dissolved in 120 ml ethanol. Sodium borohydride (0.316 g, 8.3 mmoles) was added to the solution cooled to 5°C and the mixture was stirred at room temperature for 2 h. After evaporation to dryness, 50 ml water were added and the product was extracted with diethyl ether. The ethereal phase was dried with anhydrous sodium sulfate and evaporated to dryness yielding 3.11 g of crude product 6 which was not further purified.

N-[4-hydroxybutyl]butyl[^{15}N]amine (7)

Compound 6 (2.94 g, 7.57 mmoles) was dissolved in 80 ml methylene chloride; 70% trifluoroacetic acid in water (2.56 ml) was added with vigorous stirring at 0°C, then the mixture was allowed to warm up to room temperature for 1 h with stirring. The reaction was then quenched by the addition of saturated sodium carbonate solution (14 ml). The methylene chloride layer was separated and the aqueous phase extracted again with methylene chloride. The combined organic phases were dried over anhydrous sodium sulfate, filtered and evaporated in vacuo to give the crude product 7 and the triphenylcarbinol. Product 7 was isolated by extraction into dil. HCl which was then washed with diethyl ether; after neutralization of the acidic layer, 7 could be extracted into methylene chloride. The organic solution was dried and evaporated to give the amine 7 as a clear oil (60% yield). ^{1}H NMR ($CDCl_3$) δ 3.53 (t, J = 5.0 Hz, 2H, CH_2OH); 3.51 (s, 1H, NH); 2.60 (t, J = 5.5 Hz, 2H, N-CH_2); 2.58 (t, J = 7.0 Hz, 2H, N-CH_2); 1.70-1.20 (m, 8H, 4CH_2); 0.88 (t, J = 7.2 Hz, 3H, CH_3); mass spectrum, m/z (relative intensity), 146 (2), 128 (7), 103 (57), 87 (47), 85 (100), 73 (11), 71 (12), 55 (14), 45 (31).

N-[4-hydroxybutyl]-N-[^{15}N]nitrosobutyl[^{15}N]amine (2)

Nitrosation of 7 (4.8 mmoles) was carried out as described by Okada et al. [8], using ^{15}N-sodium nitrite (6.0 mmoles) to yield compound 2 which was purified by column chromatography using 7:3 hexane-ethyl acetate as eluting solvent mixture.

The overall yield of 2 as a yellow oil was about 20%. ^{1}H NMR ($CDCl_3$) data are reported in Table 1; ^{15}N NMR, see next section. Mass spectrum, m/z (relative intensity), 176 (3.2), 159 (74), 145 (9), 127 (26), 117 (21), 101 (52), 85 (100), 71 (60), 57 (63), 55 (88), 45 (33), 43 (47), 31 (19).

RESULTS AND DISCUSSION

Before starting the preparation of the labelled nitrosamine, the feasibility of this synthesis was tested with "cold" material.

As described in Fig. 2, the first ^{15}N atom was introduced by Gabriel synthesis. ^{15}N phthalimide was condensed with triphenylmethyloxychlorobutane to yield 4. The triphenylmethyl group was used to protect the hydroxyl function and to obtain less hydrophilic products. This facilitated isolation and purification of the primary amine 5 and made it easier to work throughout the synthesis. The phthalimide group was cleaved with hydrazine, and 5 was condensed with butyraldehyde followed by sodium borohydride reduction to yield the secondary amine 6, which was not purified. After deprotection of the triphenylmethyl group the amino alcohol 7 was nitrosated as described by Okada et al. [8] with ^{15}N-sodium nitrite in hydrochloric acid medium. The final product 2 was obtained in about 20% overall yield after purification on chromatographic column. The identity of 2 was confirmed by proton and 15-nitrogen NMR and by EI and CI MS.

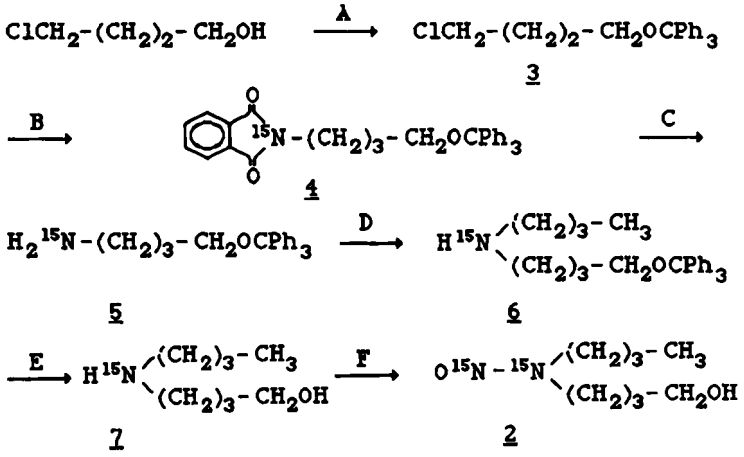

Fig. 2. Reaction scheme for the synthesis of N-[4-hydroxybutyl]-N-[^{15}N]nitrosobutyl-[^{15}N]amine (2).

A : Ph_3CCl, pyridine, room temperature.

B : ^{15}N -Potassium phthalimide, dimethylformamide, reflux.

C : $H_2NNH_2 \cdot H_2O$, ethanol, reflux.

D : 1) : $CH_3(CH_2)_2CHO$, hexane, azeotropic reflux

2) : $NaBH_4$, ethanol, 5°C.

E : CF_3COOH, methylene chloride, 5°C.

F : $Na^{15}NO_2$, conc. HCl, 5°C.

It is well known that asymmetrically substituted N-nitrosamines have complicated spectra, because of the slow dynamic equilibrium in solution between the E and Z conformers, shown in Fig. 3 [9-14].

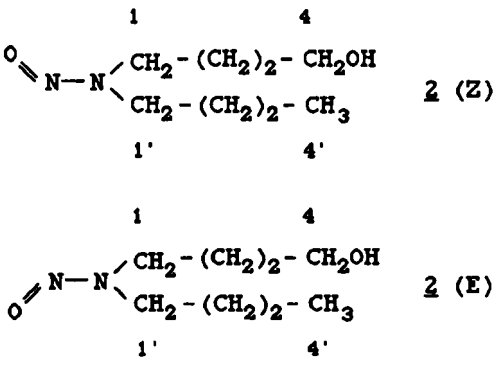

Fig. 3. E and Z conformers of N-[4-hydroxybutyl]-N-[^{15}N]nitrosobutyl[^{15}N]amine (2).

The most significant signals in the ^{1}H NMR spectra of 2 and of the corresponding unlabelled compound are summarized in Table 1. As the spectra were run at 300 MHz, all the most significant resonances are well separated for the unlabelled compound; two sets of signals can be recognized with relative intensity 85:100, which have been reasonably assigned respectively to the Z (less stable) and to the E (more stable) conformers [10]. All resonances were assigned on the basis of their multiplicity and chemical shifts, taking into account that protons resonate at higher magnetic fields when syn than when anti to the nitroso oxygen [15].

The ^{1}H NMR spectrum of compound 2 is far more complex because of further splitting of the H-1 and H-1' signals with the two active ^{15}N nuclei inside the same molecule. Strong enhancement of the resolution of the two multiplets and comparison with literature data [16] made it possible to measure and assign all ^{15}N-^{1}H coupling constants (Table 1).

The structure of compound 2 was further confirmed by ^{15}N NMR spectroscopy. The ^{1}H - coupled ^{15}N spectrum of the doubly labelled compound shows two doublets at δ 158.75 and δ -125.40 (external reference: neat CH_3NO_2) both split by a mutual coupling constant $^{1}J_{^{15}N\,-\,^{15}N}$ = 20.0 Hz. The former displays sharp lines ($\Delta\nu$ 1/2 = 5 Hz) and the latter broader ones ($\Delta\nu$ 1/2 = 12 Hz) indicating more extensive couplings with neighboring protons. Chemical shift considerations [17] and the different line widths led us to assign the lower field resonance to the nitroso and the higher field to the amino nitrogen.

TABLE 1

^1H NMR of 2 (E) *

H	1	4	1'	4'
δ	4.10	3.69	3.52	0.96
mult.	t	t	t	t
J (Hz)	7.2	6.0	7.3	7.2
mult. **	tdd	t	tt	t
J (Hz)**	$^2J_{^{15}N-^1H}$ = 1.5 $^3J_{^{15}N-^1H}$ = 1.0	6.0	$^2J_{^{15}N-^1H} \simeq$ $^3J_{^{15}N-^1H}$ = 2.7	7.2

^1H NMR of 2 (Z) *

H	1	4	1'	4'
δ	3.57	3.64	4.07	0.90
mult.	t	t	t	t
J (Hz)	7.2	6.0	7.2	7.2
mult. **	tt	t	tdd	t
J (Hz)**	$^2J_{^{15}N-^1H} \simeq$ $^3J_{^{15}N-^1H}$ = 1.0	6.0	$^2J_{^{15}N-^1H}$ = 1.8 $^3J_{^{15}N-^1H}$ = 2.7	7.2

* In $CDCl_3$. Shifts are expressed in ppm downfield from internal TMS.

** Data referred to ^{15}N doubly labelled compound.

Table 2 compares the EI fragmentation pattern of both unlabelled (1) and labelled (2) nitrosamines.

TABLE 2

	Compound 1		Compound 2	
Fragment	m/z	%	m/z	%
$[M]^+$	174	3.7	176	3.2
$[M - OH]^+$	157	55	159	74
$[M - NO]^+$	144	6	145	9
$[M - (NO + H_2O)]^+$	126	21	127	26
$[M - (CH_2)_2CH_2OH]^+$	115	17	117	21
$[M - (C_3H_7 + NOH)]^+$	100	35	101	52
$CH_3\text{-}CH_2\text{-}CH_2\text{-}CH{=}N^+{=}CH_2$ or $CH_3\text{-}CH_2\text{-}CH_2\text{-}CH_2\text{-}N^+{\equiv}CH$	84	100	85	100
$[C_4H_7O]^+$	71	47	71	60
C_4H_9	57	68	57	63
$[C_4H_7]^+$	55	99	55	88
$C_2H_6N^+$	44	40	45	33
C_3H_7	43	67	43	47
NO	30	19	31	19

The fragmentation pattern of the unlabelled nitrosamine was in agreement with previous reports [18, 19].

From comparison of the EI mass spectra of the two compounds the different fragments could be unequivocally identified. The molecular ion of 2 at m/z 176, being two mass units higher than that of the unlabelled nitrosamine, indicates that two 15-nitrogen atoms were incorporated. Moreover, all fragments containing one or two 15-nitrogen atoms showed m/z respectively one or two mass units higher, suggesting that the compound synthesized was actually 2.

The identity of compound 2 was confirmed by CI-MS. Mass spectrum analysis of purified 2 indicated 98 atom % ^{15}N incorporation. The molecular ion regions of the CI mass spectra of unlabelled and labelled nitrosamines are reported in Fig. 4a and b respectively.

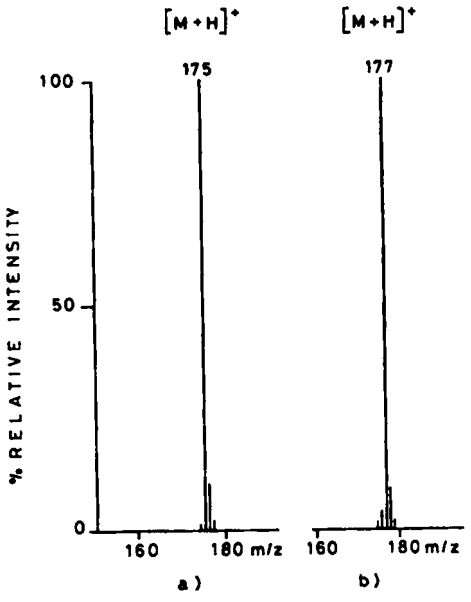

Fig. 4. CI mass spectra of 1 (a) and 2 (b).

The ability of rat liver homogenates or isolated organs to metabolize 2 to molecular $^{15}N_2$ is now being investigated.

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